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Primer Validation

Evaluating five primer pairs for environmental DNA metabarcoding of Central European fish species based on mock communities

Till-Hendrik Macher[1], Robin Schütz[1], Atakan Yildiz[2], Arne J. Beermann[1,3], Florian Leese[1,3]

1 *University of Duisburg-Essen, Aquatic Ecosystem Research, Universitaetsstr. 5, 45141 Essen, Germany*
2 *Biotechnology Institute, Ankara University, Ankara 06135, Turkey*
3 *University of Duisburg-Essen, Centre for Water and Environmental Research (ZWU), Universitaetsstr. 3, 45141 Essen, Germany*

Corresponding author: Till-Hendrik Macher (till-hendrik.macher@uni-due.de)

Abstract

Environmental DNA (eDNA) metabarcoding has become a powerful tool for examining fish communities. Prior to the introduction of eDNA-based assessments into regulatory monitoring contexts (e.g., EU Water Framework Directive), there is a demand for methodological standardization. To ensure methodical accuracy and to meet regulatory standards, various sampling, laboratory and bioinformatic workflows have been established. However, a crucial prerequisite for comprehensive fish monitoring is the choice of suitable primer pairs to accurately identify the fishes present in a given water body. Various fish-specific primer pairs targeting different genetic marker regions were published over the past decade. However, a dedicated study to evaluate the performance of frequently applied fish primer pairs to assess Central European fish species has not yet been conducted. Therefore, we created an artificial 'mock' community composed of DNA from 45 Central European fish species and examined the detection ability and reproducibility of five primer pairs. Our study highlights the effect of primer choice and bioinformatic filtering on the outcome of eDNA metabarcoding results. From the five primer pairs evaluated in our study the tele02 (12S gene) primer pair was the best choice for eDNA metabarcoding of Central European freshwater fish. Also, the MiFish-U (12S) and SeaDNA-mid (COI) primer pairs displayed good detection ability and reproducibility. However, less specific primer pairs (i.e., targeting vertebrates) were found to be less reliable and generated high numbers of false-positive and false-negative detections. Our study illustrates how the careful selection of primer pairs and bioinformatic pipelines can make eDNA metabarcoding a more reliable tool for fish monitoring.

Key words: 12S, 16S, biomonitoring, COI, eDNA

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Introduction

Environmental DNA (eDNA) metabarcoding has become a valuable tool for monitoring fish species in different habitats (McDevitt et al. 2019; Wang et al. 2021; Miya 2022). Several studies have compared eDNA-based monitoring to traditional monitoring approaches, such as gillnetting or electrofishing, proving eDNA metabarcoding to be a reliable, fast, sensitive, non-invasive, and cost-efficient method for fish detection (Pont et al. 2018; Fujii et al. 2019;

Boivin-Delisle et al. 2021). However, applying eDNA metabarcoding comes with certain challenges such as the selection of appropriate sampling strategies and wet lab processing steps, completeness of reference databases, and choice of appropriate primers (Evans et al. 2017; Kumar et al. 2022). As a prerequisite for comprehensive biodiversity monitoring, suitable primers are crucial to avoid false-negative detection and accurately depict the present fish fauna (Schenekar et al. 2020). A plethora of primer pairs suitable for eDNA metabarcoding targeting fish have been published (reviewed in Xiong et al. 2022). While some primer pairs broadly amplify eukaryotic DNA, such as the Leray-XT (Wangensteen et al. 2018) or Minibar primer pairs (Meusnier et al. 2008), other primer pairs more specifically target vertebrates (Kitano et al. 2007; Riaz et al. 2011). If the main goal is to assess the fish fauna, many primer pairs optimised to detect fish biodiversity are available. One of the most widely used universal fish primer pairs is MiFish-U (Miya et al. 2015). Based on this primer pair several, modified versions have been developed, such as the teleo (Valentini et al. 2016), elas02, and tele02 primer pairs (Taberlet et al. 2018). Many other primer pairs that either target marine (e.g., Thomsen et al. 2012; West et al. 2021) or freshwater fish species (e.g., Minamoto et al. 2012; Evans et al. 2016) can be used. In general, the choice of primers is a crucial part of planning a study as it directly depends on the target fish community.

Mock community metabarcoding is an efficient *in vitro* approach to test the performance of primer pairs using an artificially composed DNA mixture representing the expected target community (Hänfling et al. 2016; Elbrecht et al. 2019). While different metabarcoding fish primers have been evaluated on natural communities, larger systematic tests of primers with fish mock communities are missing (Bylemans et al. 2018a; Miya et al. 2020; Zhang et al. 2020; Shu et al. 2021). These studies focused on the detection of Asian and Australian fish species, which are genetically divergent and differing in species composition from the Central European fish fauna. Primer pairs for European fish communities have for now only been evaluated for estuarine and costal eDNA samples (Collins et al. 2019) and on smaller scale for UK lake fish (Hänfling et al. 2016). Thus, especially for the implementation of fish eDNA metabarcoding in routine monitoring contexts such as the European Water Framework Directive (Hering et al. 2018; Pont et al. 2021), it is crucial to evaluate suitable primer pairs AE / BE not consistent. By assessing their capability to detect present species, while minimizing false-positive and false-negative results (from here on referred to as detection ability) of the most common European freshwater fish species.

In this study, we addressed this issue and evaluated five commonly used fish eDNA metabarcoding primer pairs targeting three different barcode marker regions (12S, 16S, and cytochrome c oxidase subunit I gene) by testing their performance on an artificial community ('mock') composed of DNA from 45 Central European fish species. Specifically, we examined the detection ability and reproducibility of the five primer pairs, investigated their false-positive and false-negative detection rates, and investigated primer-specific biases. Finally, we conclude with a primer pair recommendation for eDNA metabarcoding approaches targeting fish in routine monitoring campaigns of the ichthyofauna in Central Europe.

Methods

Fish swabs

Mucus samples of 66 specimens (45 species) were collected by fish bioassessment experts during electrofishing campaigns in autumn 2020 at five sites across Germany, covering both the Rhine and the Danube catchment. Each mucus sample was collected individually using sterile swabs (FLOQ Swab 80 mm, minitip, without medium, sterile sleeve; COPAN, Italy). All fish were handled as efficiently as possible outside the water to keep the stress to a minimum, while a sterile swab was moved across the specimens' flank. Swabs were placed back into the sleeve without further preservative and sealed. After field work, samples were stored at 4 °C until delivery to the University of Duisburg-Essen. Upon arrival the swabs were stored at -20 °C overnight followed by DNA extraction the next day.

DNA extraction

Swab tips were clipped off at the handle and placed in a sterile 1.5 mL Eppendorf tube before 1 mL TNES buffer and 15 µL Proteinase K (300 U/mL, 7BioScience, Neuenburg am Rhein, Germany) were added to the sample. Samples were incubated at 55 °C and shaken at 1000 rpm for 3 h on an Eppendorf ThermoMixer C (Eppendorf AG, Hamburg, Germany). Subsequently, DNA was extracted using an adapted NucleoMag tissue kit (Macherey Nagel, Düren, Germany; Suppl. material 7). In total, a volume of 400 µL per sample was used and DNA was eluted in a final volume of 50 µL elution buffer. DNA concentration of each sample was measured using a Qubit dsDNA HS Assay-Kit on a Qubit v2 fluorometer (Thermo Fisher Scientific).

Mock community composition

Two fish mock communities were created using the extracted fish swab DNA. Both mock communities contained DNA of the same 45 fish species (Suppl. material 3). The first normalized mock community (MC1) was equimolarly pooled to 2 ng DNA per species. A second mock community (MC2) was pooled using 1 µL of each extract to generate a mock community with different DNA concentrations per species. MC2 was used to test for potential correlation between DNA concentration and number of reads. When multiple specimens were collected for a species, only the sample with the highest DNA concentration was used for the composition of the mock community in order to represent each species only by a single individual.

DNA amplification and sequencing

Both mock communities were assessed using five different published primer pairs (Table 1) for DNA amplification: tele02 (Taberlet et al. 2018), MiFish-U (Miya et al. 2015), 12Sv5 (Riaz et al. 2011), SeaDNA-mid (Collins et al. 2019), and L2513/H2714 (Kitano et al. 2007). A two-step PCR approach (Bohmann et al. 2022) was applied for amplifying the molecular marker genes and tagging of amplicons with barcodes and Illumina sequencing adaptors. In the 1st-step PCR step, tagged versions of the five fish primer pairs were used (Table 1).

Table 1. Primer pairs used for PCR amplification of the fish mock community.

Name	Gene	Primer pair	Forward sequence (5'-3')	Reverse sequence (5'-3')	Annealing temp.	Target length	Publication
tele02	12S	tele02_fw/tele02_rv	AAACTCGTGCCAGCCACC	GGGTATCTAATCCCAGTTTG	52 °C	~ 167 bp	Taberlet et al. 2018
MiFish-U	12S	MiFish-U_fw/ MiFish-U_rv	GTCGGTAAAACTCGTGCCAGC	CATAGTGGGGTATCTAATCCCAGTTTG	59 °C	~ 170 bp	Miya et al. 2015
SeaDNA-mid	COI	coi.175f/coi.345r	GGAGGCTTTGGMAAYTGRYT	TAGAGGRGGGTARACWGTYCA	53 °C	~ 130 bp	Collins et al. 2019
12SV5	12S	12S-V5f/12S-V5r	ACTGGGATTAGATACCCC	TAGAACAGGCTCCTCTAG	52 °C	~ 106 bp	Riaz et al. 2011
LH16S	16S	L2513/H2714	GCCTGTTTACCAAAAACATCA	CTCCATAGGGTCTTCTCGTCTT	55 °C	~ 220 bp	Kitano et al. 2007

In total, 60 1st-step PCR amplifications were conducted, including five replicates for each mock community (MC1 and MC2) and two negative PCR controls for each of the five primer pairs. The reaction volume was 25 µL, consisting of 12.5 µL Multiplex Mastermix (Qiagen Multiplex PCR Plus Kit, Qiagen, Hilden, Germany), 7 µL PCR-grade water, 2.5 µL CoralLoad dye, 0.5 µL forward primer, 0.5 µL reverse primer (10 µM each), and 2 µL of DNA template. The 1st-step PCR included following steps: 5 min 95 °C initial denaturation, followed by 10 cycles of 30 s at 95 °C, 90 s at decreasing annealing temperature (starting from annealing temperature +10 °C), and 30 s at 72 °C, followed by 25 cycles of 30 s at 95 °C, 90 s at the respective annealing temperature (see Table 1 for primer-specific temperatures), and 30 s at 72 °C. The final elongation was 10 min at 68 °C. Subsequently, PCR products were size selected using magnetic beads (ratio 0.7, https://doi.org/10.17504/protocols.io.36wgqj45xvk5/v2) to remove excessive primers and reduce subsequent primer dimer formation.

In the 2nd-step PCR, Illumina sequencing adapters with a dual twin-indexing system were added (Buchner et al. 2021; Bohmann et al. 2022). For each sample, the 2nd-step PCR mix contained, 7.5 µL Multiplex Mix, 1.8 µL PCR-grade water, 1.5 µL CoralLoad dye, 1.2 µL combined primer (5 µM), and 3 µL 1st-step PCR product. The 2nd-step PCR included the following steps: 5 min 95 °C initial denaturation, followed by 10 cycles of 30 s at 95 °C and 120 s at 72 °C. The final elongation was 10 min at 68 °C. The 2nd-step PCR products were visualized on a 1% agarose gel to evaluate amplification success. Then, PCR products were size selected using magnetic normalization beads (ratio 0.7, https://doi.org/10.17504/protocols.io.q26g7y859gwz/v1) to normalize sample concentration and remove excessive primers and primer dimers. Subsequently, all normalized PCR products were pooled into one library. The pooled library was concentrated using a NucleoSpin Gel and PCR Clean-up kit (Macherey Nagel, Düren, Germany) following the manufacturer's protocol. The final elution volume of the library was 40 µL. The library was then analysed using a Fragment Analyzer (High Sensitivity NGS Fragment Analysis Kit; Advanced Analytical, Ankeny, USA) to check for potential primer dimers and co-amplification, and to quantify the DNA concentration of the library. The final library was sequenced on a MiSeq 250 bp PE V3 Illumina platform at CeGat (Tübingen, Germany).

Bioinformatics

Raw reads were received as demultiplexed fastq files. All samples were processed with the APSCALE-GUI pipeline v1.1.6 (Buchner et al. 2022), which is based on VSEARCH (Rognes et al. 2016) and cutadapt (Martin 2011). Each primer pair was processed separately. All settings were kept as default

(maxdiffpct = 25, maxdiffs = 199, minovlen = 5; maxEE = 1; min size to pool = 4), and OTUs were clustered with a 97% percentage similarity threshold. For the ribosomal genes 12S and 16S that have a somewhat lower mutation rate compared to COI we also used a more stringent clustering threshold of 99%. As this did not substantially alter the results, we used a single workflow subsequently. Subsequently, taxonomy was assigned using the 'local BLAST' function in APSCALE with the Midori2 databases (v249 of CO1, lrRNA for 16S and srRNA for 12S; Leray, Knowlton, and Machida 2022) as reference (default word size 11).

The taxonomic assignment of each OTU was filtered using APSCALE-GUI (Fig. 1). Initially, taxonomic assignments were filtered by e-value (hits with the lowest e-value are kept) and hits with the same taxonomy were dereplicated. Subsequently, assignment of taxonomic levels was done using a similarity threshold approach (species ≥ 97%, genus ≥ 95%, family ≥ 90%, order ≥ 85%). If at this point more than one taxon assigned to species level remained, additional filtering and flag raising steps were performed as follows: All ambiguous taxa were saved to a separate column in the taxonomy table. The number

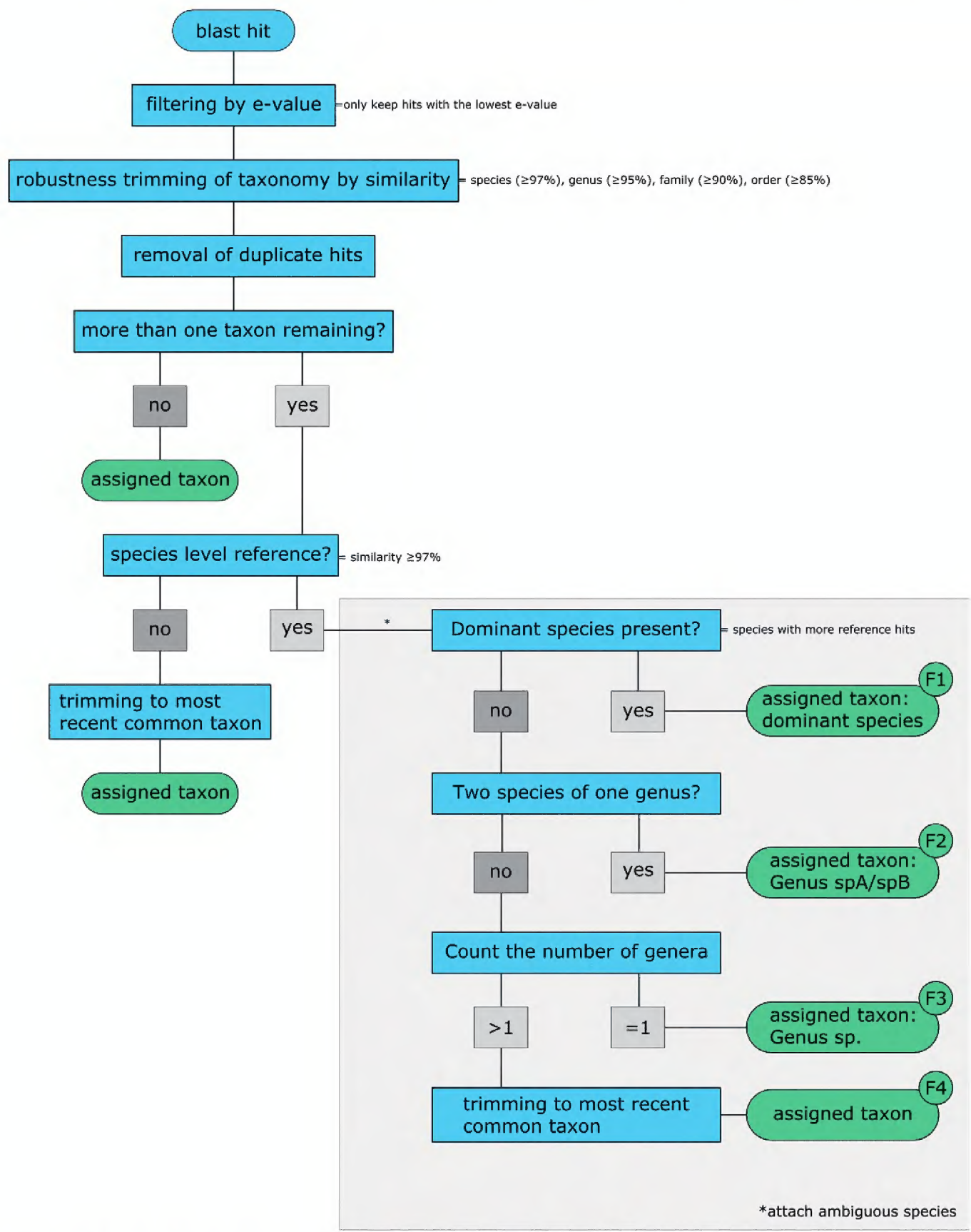

Figure 1. Decision tree for taxonomic assignment implemented in APSCALE-GUI v1.2.0.

of occurrences per remaining taxon was counted. If a dominant species was present, it was selected for taxonomic assignment ("F1 – Dominant taxon"). Otherwise, if two species of the same genus remained, the genus was saved with the two possible species names separated by slash (e.g., *Leuciscus idus/leuciscus*; "F2 – Two species, one genus"). If more than two species belonging to different genera remained, the number of genera was counted. If one genus (and multiple species) was present the genus was saved (e.g., *Hucho* sp. with the ambiguous assignments *Hucho bleekeri*, *Hucho hucho*, and *Hucho taimen*; "F3 – Multiple species of one genus"). Lastly, if more than one genus remained and no dominant taxon was present, the taxonomic assignment was trimmed to the most recent common taxon ("F4 – Multiple genera"). Both the taxonomy and read tables were then converted to TaXon tables (Suppl. material 8) for downstream analyses in TaxonTableTools v1.4.7 (TTT, Macher et al. 2021a). To account for potential contamination the sum of reads in the negative controls of each OTU was subtracted from the number of reads for the respective OTU of each sample ('Negative control subtraction' tool). Subsequently, all tables were filtered for fish and lamprey species (Suppl. material 9). Here, all OTUs with a ≥97% similarity but without species assignment were manually checked and adjusted if e.g., a hybrid or erroneous entry was preventing a species assignment (Suppl. material 10). If the taxonomy was ambiguous due to the assignment to geographically clearly separated species with equal similarity values, the species which is reported from the area was selected. The distribution information was collected from the gbif database (www.gbif.org).

Analyses were performed using custom python scripts (Suppl. material 11) and results were visualized using the plotly package https://plot.ly. For all primer pairs, the OTU and read proportions of target taxa (i.e., fish and lamprey) and bycatch taxa (i.e., all other taxa) were calculated. Additionally, the number of ambiguous species-level OTUs and the number of occurrences of each flag was calculated. For all subsequent analyses the manually adjusted TaXon tables were used.

Statistical analyses

First, the relative read abundances (%) for the target species (i.e., fish) and non-target species (bycatch) present in the mock were calculated for each primer pair. Additionally, the relative OTU proportions of target and bycatch species were calculated. Also, the proportions of species-level OTUs assigned to the four flags (F1–F4) and supported species were calculated. All three analyses were displayed as bar charts.

Second, to assess each primer pair's detection abilities, Venn diagrams comparing the detected species of each primer pair to the original fish mock community composition were created in TTT. Additional Venn diagrams were created to compare the pre-adjusted TaXon tables.

Third, the log transformed number of reads and the log transformed DNA concentration (ng/µL) were plotted and Pearson coefficients were calculated. Also, the log transformed number of reads per species of MC2 were plotted against the log transformed reads per species of MC1 and a Pearson coefficients was calculated.

Additionally, oversplitting rates (i.e., number of additional OTUs) were calculated for all species and each primer pair. Also, PCR replicates were investigated

by calculating the mean, minimum, and maximum Jaccard index of all five technical replicates per primer pair.

To estimate the completeness of our fish mock community, we downloaded all freshwater fish species reported from Germany and their occurrence categories per fishbase.org (categories: “endemic”, “introduced”, “native”, “not established”, “questionable”, and “stray”).

To investigate the reference sequence coverage for the fish species present in the mock community, the three Midori2 databases used for taxonomic assignment were searched for the respective species and all their records were extracted into three separate fasta files per species (COI, lrRNA, and srRNA). Subsequently, cutadapt was used to search all four possible combinations (considering reverse complements) of each primer pair. An error rate of 0.3 (-e 3) was allowed and the primers were required to be linked (forward...reverse). Here, each detected barcode was only counted once (in the four possible combinations). Consequently, for each of the three markers used, the overall number of available reference sequences, the number of matches per primer pair, and its respective proportion were calculated for all fish species in the mock community. Since in some cases only the target fragment without the primer binding site was uploaded as reference sequence, cutadapt consequently reported false-negative results to a certain degree. Thus, we manually checked all cases for which at least one reference sequence was found per species and marker, but no primer pair match was observed.

Results

Fish mock community composition

According to the fishbase database, 123 freshwater fish species are reported from Germany. Here we manually added the round goby (*Neogobius melanostomus*) and the rainbow trout (*Oncorhynchus mykiss*), as they are both non-native species reported from Germany, but were not present in the fishbase list. Consequently, our fish mock community of 45 Central European freshwater fish species represents about 36.6% of fish reported from Germany (Suppl. material 4). In detail, our mock community accounts for 50% of “native”, 26% of “introduced”, 22.2% of “questionable”, and 8% of “not established” fish species in Germany.

DNA extraction, sequencing, and bioinformatics

DNA was successfully extracted from 66 swabs, yielding an average DNA concentration of 5.73 ng/µL, ranging from 0.05 ng/µL to 26.3 ng/µL (Suppl. material 3). Sequencing of mock community library yielded a total of 8,254,293 raw reads across all primer pairs. In total, 7,745,593 quality-filtered reads were clustered into 140 (tele02), 105 (MiFish-U), 120 (12SV5), 111 (SeaDNA-mid), and 142 (LH16S) OTUs, respectively. In total, 4379 reads were detected in the negative controls (tele02: 22 reads, MiFish: 19, 12SV5: 4338, SeaDNA-mid: 0, and LH16S: 0), which were then subtracted. Nearly all primer pairs showed little amplification of non-fish OTUs (between 96 to 98% fish OTUs), except for the SeaDNA-mid primer pair, which exhibited 50% non-fish OTUs, (Fig. 2A). However, only few reads were assigned to non-target OTUs for all primer pairs (between 98.2 and 100% fish OTUs; Fig. 2B). The proportions of flagged taxonomic assignments varied between the

five different primer pairs. Here, both the MiFish-U and tele02 primer pairs had the highest proportion of supported species-level OTUs (both 60%), followed by the SeaDNA-mid (49%), 12SV5 (46%), and LH16S (41%) primer pairs (Fig. 2C). The flag 'Two species, one genus' (Flag 2) was most prominent in the SeaDNA-mid (25%) and least prominent in the 12SV5 primer pair (10%). For the flag 'Multiple species of one genus' (Flag 3) again the SeaDNA-mid showed the highest proportions (16%), while both the tele02 and MiFish-U primer pairs had the fewest cases (5%). Furthermore, the 12SV5 primer pair showed the highest proportion of the flag 'Dominant taxon' (Flag 1) with 27% assigned species-level OTUs, while again the tele02 primer pair showed the fewest (9%). The SeaDNA-mid primer did not have any cases of flag 'Multiple genera' (Flag 4), while the LH16S primer pair had the most (9%). Overall, the most abundant ambiguous assignment was *Leuciscus idus/leuciscus* (10 total occurrences), followed by *Sander canadensis/lucioperca* (8), *Blicca bjoerkna* (7), *Proterorhinus semilunaris/marmoratus* (6), and

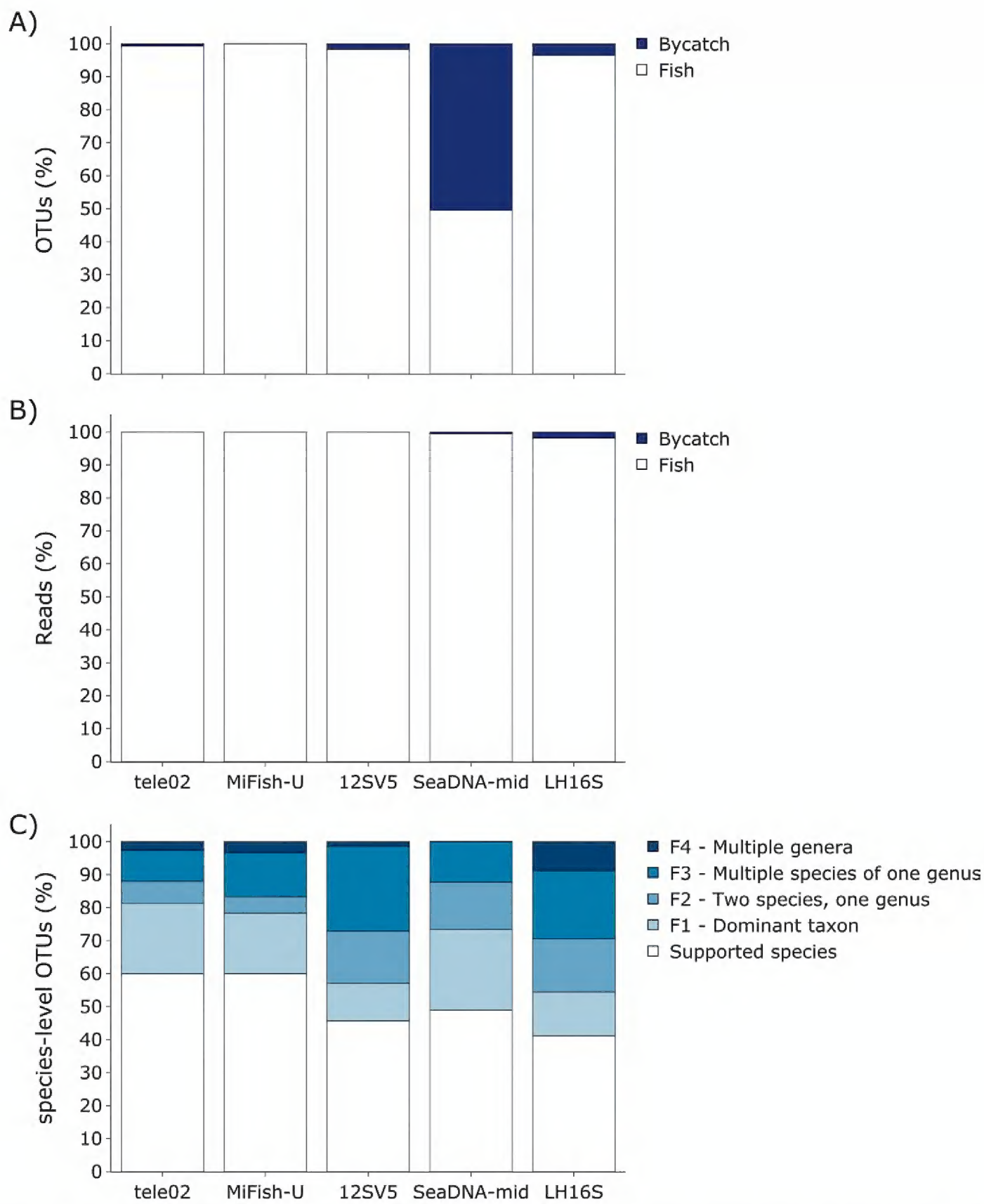

Figure 2. Proportions of fish and non-fish OTUs (**A**) and read proportions (**B**) detected with the five different primer pairs (**A**), and the proportions of ambiguous taxonomic assignments (flags 1–4) for all species-level OTUs (**C**), based on the pre-adjusted datasets.

Cyprinus carpio and *Hucho* sp. with each 5 cases (Suppl. material 5). Overall, the genera *Leuciscus* and *Sander* showed the highest number of ambiguous taxonomic assignments (14 and 13, respectively).

Taxonomic composition comparison

After removal of bycatch taxa and curation of ambiguous taxonomic assignments, the 12SV5 primer pair (45) included most species, followed by LH16S (40), tele02 (39 species), MiFish-U (37), and SeaDNA-mid (36). In comparison to the original mock community fish species composition, the tele02 dataset showed the highest congruence (2 false-positive species, 37 true positive, and 8 false-negative), followed by the MiFish-U (2, 35, 10) and SeaDNA-mid (3, 33, 12). Both the 12SV5 (18, 27, 18) and LH16S primer pair (17, 23, 22) were less congruent to the original mock community composition (Fig. 4). The 12SV5 and LH16S primer pairs resulted in OTUs assigned to several marine fish taxa, which were not part of the mock community, including Acanthuridae (surgeon fishes), Kyphosidae (sea chubs), Ophidiidae (cusk-eel), Peristediidae (armoured sea robins), Pholidae, and Zoarcidae (eelpouts; Fig. 3A, B). Regarding the number of false-positive and false-negative assignments per family, the LH16S primer pair showed high incongruences to the mock community, particularly for the Leuciscidae (4 false-positive / 10 false-negative) and Percidae (2/2). Similarly, the 12SV5 primer pair had various false-positive and false-negative assignments for the Leuciscidae (6/5), Cyprinidae (4/0), or Gobionidae (3/1). The SeaDNA-mid primer showed only a moderate number of incorrect assignments in the Leuciscidae (2/6). Lastly, the tele02 and MiFish-U primer pairs were overall the least prone to false-positive assignments and only showed false-positive assignments in Leuciscidae (*Leuciscus aspius*) and Salmonidae (*Parahucho perryi* and *Brachymystax lenok*).

Primer bias impact on species detection

As a measure of primer bias the standard deviation of relative read abundances was across primer pairs. Here the standard deviation varied between the primer pairs ranging from an average of < 0.01% (*Barbatula barbatula*, *Leucaspius delineatus*, *Neogobius melanostomus*, *Phoxinus phoxinus*, and *Romanogobio albipinnatus*) to a maximum of 7.5% (*Pungitius pungitius*; Table 2A). While most species were detected with at least four primer pairs (29 mock community species), 10 species were detected with three or less primer pairs. In total, six species were not detected by any of the primer pairs, namely *Cottus gobio*, *Gymnocephalus schraetser*, *Lampetra fluviatilis*, *Rutilus pigus*, *Umbra krameri*, and *Zingel zingel*. Most false-positive species were unique to one primer pair (34 of 37 species; Table 2B), while only three species were detected with two or more primer pairs, namely *Leuciscus aspius* (4 occurrences), *Pungitius platygaster* (2), and *Umbra pygmaea* (2).

Evaluation of oversplitting rates

In total, 48 cases of oversplitting (in our case species with more than one OTU assigned) were observed (Suppl. material 6). Most oversplit species-level assigned OTUs were found with the tele02 primer pair (12), while all other primer

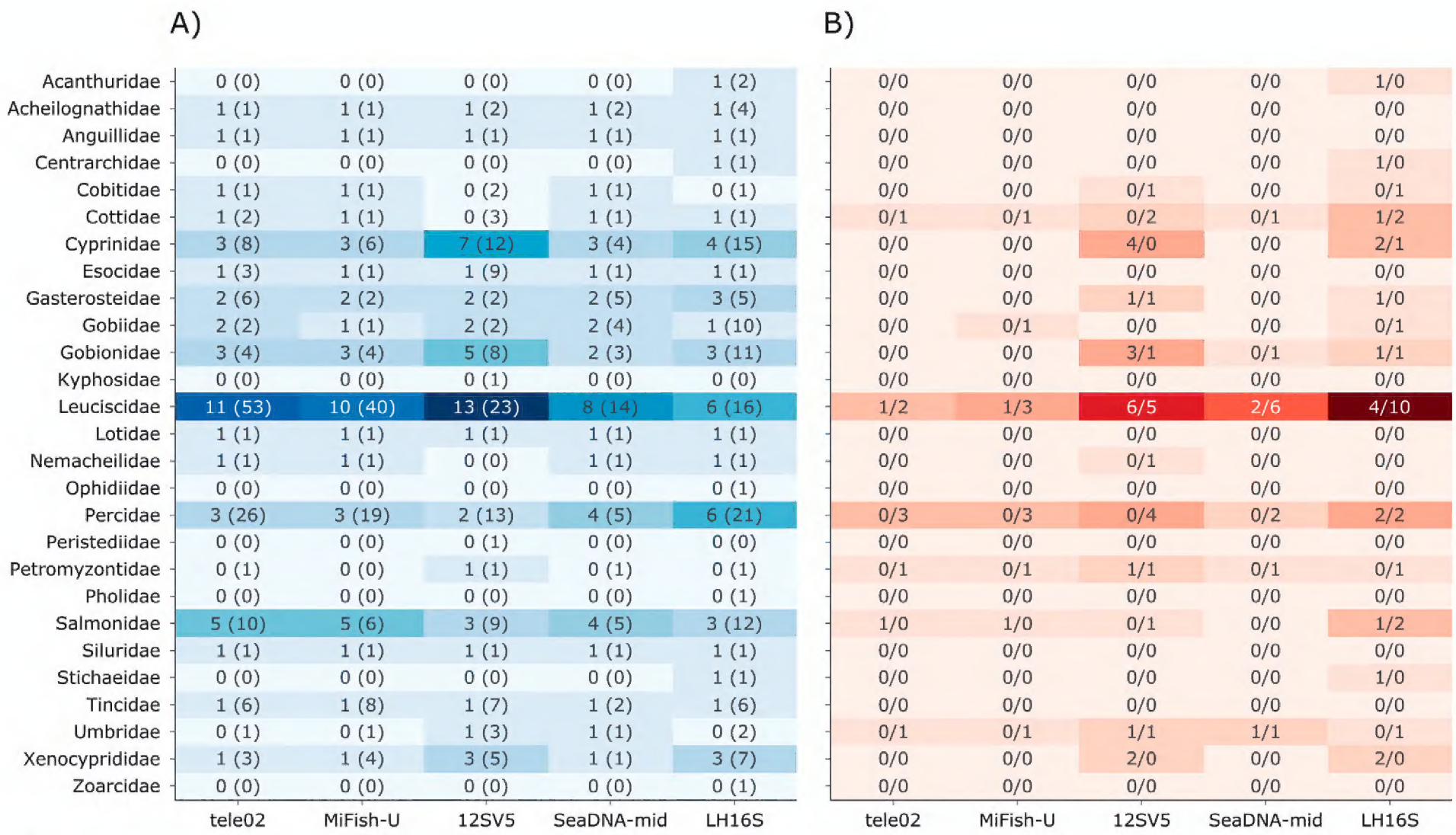

Figure 3. A Overall number of fish species and the respective number of OTUs (in brackets) per family detected in the mock community for each primer pair. **B** Number of false-positive (n/) and false-negative (/n) fish species detections compared to the original fish mock community composition.

pairs showed nine cases of oversplitting. The highest oversplitting rate was observed in *Gymnocephalus cernua* (7-fold OTU to species ratio, tele02 primer pair) and *Tinca tinca* (7-fold, 12SV5). While no over-split species was found in all five or even four of the primer pairs, six species were over split in three primer pairs (i.e., *Abramis brama*, *Blicca bjoerkna*, *Ctenopharyngodon idella*, *Gymnocephalus cernua*, *Hucho hucho*, and *Sander lucioperca*).

PCR replicate consistency assessment

PCR replicates were highly consistent for all investigated primer pairs. The 12SV5 primer pair showed the highest reproducibility (mean Jaccard similarity of 0.99), followed by LH16S (0.98), SeaDNA-mid (0.96), tele02 (0.96), and MiFish-U (0.95). No correlations between log transformed input DNA concentration (ng/µL) and log transformed reads of the second mock community (MC2) were found for all primer pairs (Pearson correlation between 0.12 and 0.16, $p \geq 0.05$; Suppl. material 1). However, when comparing the number of log transformed reads per species between MC1 and MC2, significant Pearson correlations for the tele02 (0.8, $p \leq 0.05$), MiFish-U (0.79, $p \leq 0.05$), 12SV5 (0.8, $p \leq 0.05$), SeaDNA-mid (0.81, $p \leq 0.05$), and LH16S (0.68, $p \leq 0.05$) primer were found (Suppl. material 2).

Reference database coverage

The Midori2 v249 reference database assessment showed that for COI markers most reference sequences are available (2313), followed by the lrRNA

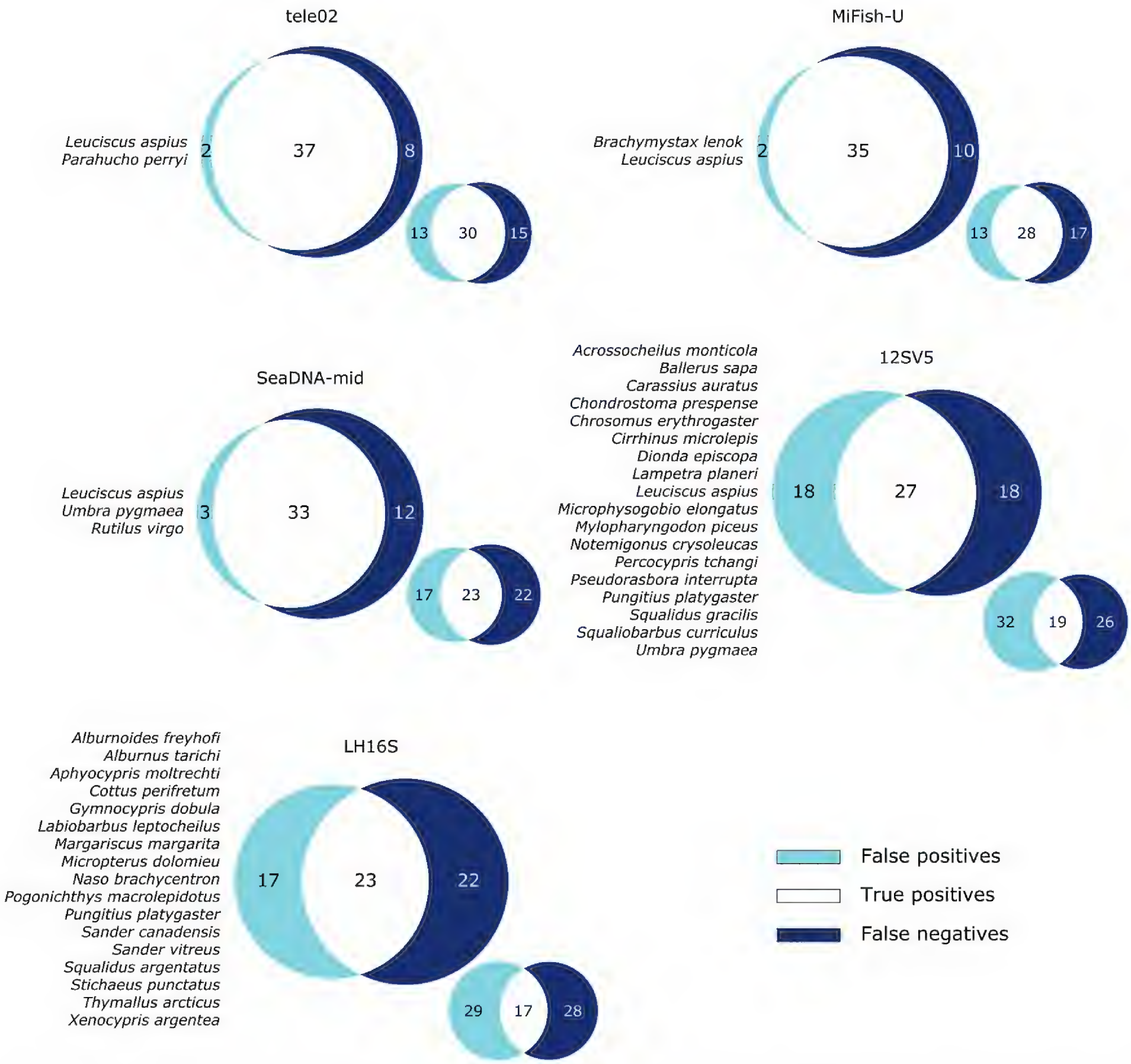

Figure 4. Comparison of the fish mock community species composition to the detected species with each primer pair for both the adjusted (large Venn diagrams) and the pre-adjusted datasets (small Venn diagrams). All species declared as false-positive detections are listed on the left-hand side of the respective Venn diagram.

(809), and srRNA (379) markers (Table 3). In detail, the SeaDNA-mid (COI) primer showed the highest number of matches within the available reference sequences (avg. 96.85% coverage) Only one species did not possess a reference sequence (i.e., *Romanogobio albipinnatus*) for COI. The LH16S primer also showed a high coverage (avg. 91.84%). Here, two species did not have a reference sequence available (i.e., *Romanogobio albipinnatus* and *Rutilus pigus*). On the other hand, the srRNA coverage was drastically lower for all three investigated primer pairs. The 12SV5 and tele02 primer pairs showed slightly higher coverage (avg. 77.75% and 76.06%) compared to the MiFish-U primer pair (avg. 66.05%). Four species in total did not have a match for any on the srRNA primer pairs (i.e., *Gymnocephalus schraetser*, *Rutilus pigus*, *Zingel streber*, and *Zingel zingel*). Another two species were only detected with one of the srRNA primer pairs (i.e., *Misgurnus fossilis* and *Umbra rameria*).

Table 2. Relative read abundances (%) for all detected fish and lamprey species of all five primer pairs, **A**) including all species present in the mock community (i.e., true positive species) and **B**) all non-target species (i.e., false-positive species). For each species the number of positive detections (occurrences) and the standard deviation (STDEV) were calculated.

True positive species	tele02	MiFish-U	12SV5	SeaDNA-mid	LH16S	Occurrences	STDEV	*False positive species*	tele02	MiFish-U	12SV5	SeaDNA-mid	LH16S	Occurrences	STDEV
			A								B				
Anguilla anguilla	0.103	0.095	0.119	0.002	0.147	5	0.1	*Leuciscus aspius*	0.512	0.556	0.091	0.016	0	4	0.3
Silurus glanis	0.129	0.02	0.164	0.15	0.064	5	0.1	*Pungitius platygaster*	0	0	0.008	0	0.002	2	0.0
Barbus barbus	0.306	0.341	0.544	0.775	0.635	5	0.2	*Umbra pygmaea*	0	0	1.002	0.005	0	2	0.7
Thymallus thymallus	0.466	0.507	0.731	0.033	1.208	5	0.4	*Acrossocheilus monticola*	0	0	0.013	0	0	1	
Tinca tinca	3.682	4.099	4.393	4.852	4.523	5	0.4	*Alburnoides freyhofi*	0	0	0	0	37.686	1	
Gymnocephalus cernua	2.243	2.241	2.37	0.357	1.108	5	0.9	*Alburnus tarichi*	0	0	0	0	0.146	1	
Ctenopharyngodon idella	1.15	0.153	1.39	2.576	0.667	5	0.9	*Aphyocypris moltrechti*	0	0	0	0	0.35	1	
Lota lota	0.264	0.253	0.329	3.746	0.191	5	1.6	*Ballerus sapa*	0	0	0.922	0	0	1	
Gobio gobio	0.179	0.192	0.24	3.816	0.141	5	1.6	*Brachymystax lenok*	0	0.005	0	0	0	1	
Rhodeus sericeus/amarus	1.46	1.412	1.544	5.838	0.73	5	2.1	*Carassius auratus*	0	0	0.031	0	0	1	
Carassius carassius	2.366	2.539	3.167	7.149	2.121	5	2.1	*Chondrostoma prespense*	0	0	2.083	0	0	1	
Rutilus rutilus	2.502	2.558	0.353	1.746	6.3	5	2.2	*Chrosomus erythrogaster*	0	0	0.028	0	0	1	
Esox lucius	6.232	5.7	5.131	0.049	0.432	5	3.0	*Cirrhinus microlepis*	0	0	0.017	0	0	1	
Perca fluviatilis	1.485	1.321	12.65	0.18	1.174	5	5.2	*Cottus perifretum*	0	0	0	0	0.964	1	
Proterorhinus semilunaris	0.267	0.007	0.007	7.917	11.719	5	5.5	*Dionda episcopa*	0	0	0.118	0	0	1	
Hucho hucho	2.067	2.288	2.494	17.357	3.042	5	6.7	*Gymnocypris dobula*	0	0	0	0	0.05	1	
Pungitius pungitius	0.491	0.58	0.677	17.491	0.839	5	7.5	*Labiobarbus leptocheilus*	0	0	0	0	0.02	1	
Phoxinus phoxinus	0.003	0.002	0.003	0	0.005	4	0.0	*Lampetra planeri*	0	0	0.004	0	0	1	
Barbatula barbatula	0.003	0.004	0	0.006	0.002	4	0.0	*Margariscus margarita*	0	0	0	0	0.24	1	
Oncorhynchus mykiss	0.149	0.192	0.294	0.54	0	4	0.2	*Microphysogobio elongatus*	0	0	0.007	0	0	1	
Cyprinus carpio	0.372	0.363	0.506	0.019	0	4	0.2	*Micropterus dolomieu*	0	0	0	0	0.284	1	
Gasterosteus aculeatus	0.197	0.218	0	0.732	0.25	4	0.3	*Mylopharyngodon piceus*	0	0	0.057	0	0	1	
Squalius cephalus	1.072	1.09	0.09	0.007	0	4	0.6	*Naso brachycentron*	0	0	0	0	0.687	1	
Pseudorasbora parva	0.214	0.212	0	1.972	0.206	4	0.9	*Notemigonus crysoleucas*	0	0	0.159	0	0	1	
Chondrostoma nasus	2.333	2.508	0.047	0.408	0	4	1.3	*Parahucho perryi*	0.007	0	0	0	0	1	
Blicca bjoerkna	5.043	5.176	0.161	1.269	0	4	2.6	*Percocypris tchangi*	0	0	0.046	0	0	1	
Abramis brama	19.839	19.776	25.78	17.748	0	4	3.5	*Pogonichthys macrolepidotus*	0	0	0	0	0.226	1	

True positive species	tele02	MiFish-U	12SV5	SeaDNA-mid	LH16S	Occurrences	STDEV	*False positive species*	tele02	MiFish-U	12SV5	SeaDNA-mid	LH16S	Occurrences	STDEV
			A								B				
Alburnus alburnus	6.532	6.837	10.588	1.189	0	4	3.9	*Pseudorasbora interrupta*	0	0	0.393	0	0	1	
Sander lucioperca	8.895	8.623	0	0.054	3.856	4	4.2	*Rutilus virgo*	0	0	0	0.522	0	1	
Romanogobio albipinnatus	0.003	0.005	0.006	0	0	3	0.0	*Sander canadensis*	0	0	0	0	0.01	1	
Neogobius melanostomus	0.003	0	0.004	0.026	0	3	0.0	*Sander vitreus*	0	0	0	0	0.059	1	
Salmo trutta	0.01	0.01	0	0.2	0	3	0.1	*Squalidus argentatus*	0	0	0	0	0.034	1	
Misgurnus fossilis	0.039	0.038	0	0.821	0	3	0.5	*Squalidus gracilis*	0	0	0.091	0	0	1	
Cottus rhenanus	1.065	1.029	0	0.005	0	3	0.6	*Squaliobarbus curriculus*	0	0	0.071	0	0	1	
Leucaspius delineatus	0.002	0.001	0	0	0	2	0.0	*Stichaeus punctatus*	0	0	0	0	14.238	1	
Zingel streber	0	0	0	0.158	0.333	2	0.1	*Thymallus arcticus*	0	0	0	0	0.157	1	
Leuciscus idus	0	0.131	0	0	0	1		*Xenocypris argentea*	0	0	0	0	0.047	1	
Leuciscus leuciscus	0.053	0	0	0	0	1									
Scardinius erythrophthalmus	0.07	0	0	0	0	1									
Cottus gobio	0	0	0	0	0	0									
Gymnocephalus schraetser	0	0	0	0	0	0									
Lampetra fluviatilis	0	0	0	0	0	0									
Rutilus pigus	0	0	0	0	0	0									
Umbra krameri	0	0	0	0	0	0									
Zingel zingel	0	0	0	0	0	0									

Table 3. Assessment of the Midori2 reference database (v249) coverage for the species present in the fish mock community. The overall number of reference sequences per maker region (COI, lrRNA for 16S, and srRNA for 12S) and the number and percentage of matching reference sequences for each primer are shown.

Species	COI			lrRNA			srRNA						
	References	SeaDNA-mid	SeaDNA-mid (%)	References	LH16S	LH16S (%)	References	12SV5	12SV5 (%)	MiFish-U	MiFish-U (%)	tele02	tele02 (%)
Abramis brama	34	34	100	6	6	100	7	6	85.71	6	85.71	7	100
Alburnus alburnus	59	59	100	9	9	100	3	3	100	2	66.67	2	66.67
Anguilla anguilla	169	168	99.41	165	146	88.48	26	25	96.15	23	88.46	24	92.31
Barbatula barbatula	48	47	97.92	10	10	100	7	6	85.71	6	85.71	7	100
Barbus barbus	25	25	100	4	4	100	4	3	75	2	50	3	75
Blicca bjoerkna	25	25	100	9	9	100	3	3	100	3	100	3	100
Carassius carassius	23	23	100	8	7	87.5	5	5	100	4	80	4	80
Chondrostoma nasus	35	34	97.14	4	4	100	3	2	66.67	2	66.67	2	66.67
Cottus gobio	27	27	100	5	4	80	2	2	100	2	100	2	100
Cottus rhenanus	10	10	100	1	1	100	1	1	100	1	100	1	100
Ctenopharyngodon idella	57	56	98.25	23	23	100	12	9	75	11	91.67	12	100
Cyprinus carpio	261	246	94.25	110	98	89.09	72	62	86.11	50	69.44	54	75
Esox lucius	65	65	100	21	21	100	12	11	91.67	4	33.33	4	33.33
Gasterosteus aculeatus	93	92	98.92	23	20	86.96	23	18	78.26	11	47.83	16	69.57
Gobio gobio	26	26	100	11	10	90.91	4	3	75	3	75	4	100
Gymnocephalus cernua	40	40	100	9	9	100	6	6	100	4	66.67	4	66.67
Gymnocephalus schraetser	5	5	100	1	1	100	0	0	0	0	0	0	0
Hucho hucho	12	12	100	3	3	100	1	1	100	1	100	1	100
Hucho taimen	20	20	100	6	6	100	1	1	100	1	100	1	100
Lampetra fluviatilis	22	21	95.45	7	7	100	1	1	100	1	100	1	100
Leucaspius delineatus	25	24	96	6	6	100	1	1	100	1	100	1	100
Leuciscus idus	28	28	100	7	7	100	4	3	75	2	50	3	75
Leuciscus leuciscus	59	57	96.61	29	29	100	3	1	33.33	1	33.33	3	100
Lota lota	44	42	95.45	15	14	93.33	11	10	90.91	9	81.82	9	81.82
Misgurnus fossilis	12	12	100	2	1	50	1	0	0	0	0	1	100
Neogobius melanostomus	45	45	100	5	5	100	7	6	85.71	5	71.43	5	71.43
Oncorhynchus mykiss	124	123	99.19	36	31	86.11	23	18	78.26	16	69.57	17	73.91
Perca fluviatilis	62	62	100	35	35	100	13	11	84.62	7	53.85	8	61.54
Phoxinus phoxinus	177	176	99.44	17	17	100	13	11	84.62	11	84.62	13	100
Oxyeleotris marmorata	17	17	100	5	5	100	6	5	83.33	4	66.67	5	83.33
Pseudorasbora parva	108	108	100	37	35	94.59	13	9	69.23	9	69.23	13	100
Pungitius pungitius	61	56	91.8	40	40	100	21	20	95.24	15	71.43	16	76.19
Rhodeus amarus	27	27	100	4	4	100	1	1	100	1	100	1	100
Rhodeus sericeus	10	10	100	1	1	100	3	2	66.67	1	33.33	2	66.67
Romanogobio albipinnatus	0	0	0	0	0	0	1	1	100	1	100	1	100
Rutilus pigus	2	2	100	0	0	0	0	0	0	0	0	0	0
Rutilus rutilus	56	55	98.21	12	12	100	7	6	85.71	3	42.86	3	42.86
Salmo trutta	143	142	99.3	57	52	91.23	19	17	89.47	14	73.68	16	84.21

Species	COI			lrRNA			srRNA						
	References	SeaDNA-mid	SeaDNA-mid (%)	References	LH16S	LH16S (%)	References	12SV5	12SV5 (%)	MiFish-U	MiFish-U (%)	tele02	tele02 (%)
Sander lucioperca	28	28	100	7	7	100	5	3	60	5	100	5	100
Scardinius erythrophthalmus	30	30	100	8	7	87.5	5	4	80	3	60	4	80
Silurus glanis	25	25	100	6	6	100	2	2	100	2	100	2	100
Squalius cephalus	62	60	96.77	8	8	100	4	4	100	3	75	3	75
Thymallus thymallus	49	49	100	22	20	90.91	16	15	93.75	15	93.75	15	93.75
Tinca tinca	48	47	97.92	9	9	100	6	5	83.33	4	66.67	5	83.33
Umbra krameri	2	2	100	1	1	100	1	1	100	0	0	0	0
Zingel streber	9	9	100	3	3	100	0	0	0	0	0	0	0
Zingel zingel	4	4	100	2	2	100	0	0	0	0	0	0	0

Discussion

Detection ability, and reproducibility

Our primer evaluation based on mock communities of 45 European freshwater fish species confirmed the previously reported high detection ability for two primer pairs (MiFish-U and tele02) belonging to the MiFish primer group (Bylemans et al. 2018a; Taberlet et al. 2018; Collins et al. 2019; Polanco et al. 2021). The tele02 primer pair (a modified version of the MiFish-U primer pair) performed particularly well in our study and clearly showed the highest species specificity and detection ability for European freshwater species. Until now the tele02 primer pair was evaluated *in silico* (Taberlet et al. 2018; Collins et al. 2019) as well as for water samples from Beijing, where it exhibited outstanding detection success of fish diversity in comparison with other fish-specific primers tested (Zhang et al. 2020). Accordingly, our results show that the tele02 primer pair recovered most true-positive species while producing the lowest number of false-positive and negative detections. From all primer pairs tested in this and other studies, the tele02 primer pair is arguably the best currently available choice for fish eDNA metabarcoding of European freshwater fish. Its only caveat might be the highest observed oversplitting rate of the investigated primer pairs. While this does not affect the analysis when working on species level (i.e., OTUs of the same species are merged), the alpha diversity is artificially inflated. Here we observed that particularly the Leuciscidae showed drastically higher numbers of OTUs than species. If the analysis of OTUs is of particular interest, this issue can be tackled by e.g., using a post-clustering curation algorithm, such as LULU filtering (Frøslev et al. 2017), which should give more reliable biodiversity estimates e.g., when taxonomic references are lacking. However, this is not of further concern when working with the taxonomic assignment, since OTUs with unique taxonomic assignments can simply be merged. While the SeaDNA-mid primer pair, targeting the COI gene, showed comparable good detection ability (i.e., true-positive detections), the co-amplification of non-fish taxa with this primer pair might be of concern. The fish mucus likely accumulates eDNA molecules and thus also contains DNA from other organisms than the fish itself. Here, the SeaDNA-mid primer pair was the only primer pair that showed high numbers of non-target OTUs (e.g., Annelida, Arthropoda, Bacillariophyta, Chlorophyta, Oomycota, and Rotifera). While non-target OTUs were observed in low read abundances for the mock communities, co-amplification issues could be more pronounced when

applying the SeaDNA-mid primer pair on environmental samples. Here, comparably deeper sequencing depths might be required to detect all present fish species in an environmental sample with more non-target DNA, which would reduce the cost-efficiency per sample. The remaining two primer pairs 12SV5 and LH16S were designed to generally amplify vertebrate DNA (Kitano et al. 2007; Riaz et al. 2011; Hänfling et al. 2016; Harper et al. 2019). We decided to include these primer pairs since they have the potential for more holistic monitoring approaches, e.g., targeting the whole vertebrate community associated to a freshwater habitat (Pertoldi et al. 2021; Dou et al. 2023). However, the broader target range resulted in a drastically lower detection rate of fish.

Overall, all primer pairs generated highly reproducible taxa lists among the PCR replicates for the mock fish communities. However, this might not be achieved for environmental samples in which a lower reproducibility is expected. Therefore, sufficient field and laboratory replicates to maximize species detection and minimize stochastic sampling effects are recommended (Sato et al. 2017; Bylemans et al. 2018b; Macher et al. 2021b; Rojahn et al. 2021). Particularly the SeaDNA-mid primer pair might suffer from lower reproducibility for environmental samples due to the strong co-amplification.

Read proportions

Independent fish mucus samples used for DNA extraction are likely to contain different proportions of target fish species DNA and other, non-target DNA (e.g., of microbes). Solely for this reason, differences in relative read abundances between the 45 species of the used mock community were already to be expected. In contrast, different read proportions between primer pairs within one species were unexpected, as all used marker regions are mitochondrial and hence present in an equal copy number. However, several species showed overproportional read abundances for one of the used primer pairs, such as *Perca fluviatilis* (12SV5: 12.65% to an average of other primer pairs of 1.04%), *Hucho hucho* (SeaDNA-mid: 17.36% to 2.5%), or *Pungitius pungitius* (SeaDNA-mid: 17.49 to 0.65%). The observed differences in read proportion hint at different primer binding efficiencies for the tested primer pairs and species present in the mock community. As a consequence, the primer choice can have direct implications when using read abundances as a proxy for fish biomass (Takahara et al. 2012; Kelly et al. 2019; Muri et al. 2020). While trends between read abundances and biomass might exist, the interpretation of reads as proxy for biomass should be taken with caution and be interpreted with respect of the characteristics of the chosen primer pair.

False-negative assignments

The Midori2 database is a curated version of the larger GenBank database and can be used as a reliable source for taxonomic assignment of fish OTUs. All species present in the mock community have reference sequences available for at least one genetic marker. However, seven species were not detected at all.

Amongst these was *Cottus gobio*, a common fish species in Central Europe for which 34 reference sequences comprising all three investigated markers are deposited in Midori2 v249. Although a taxonomic assignment was possible,

no primer pair detected *C. gobio* in the mock communities. Since this species is frequently detected with eDNA metabarcoding from various sites and samples (Macher et al. unpublished data; tele02 primer pair), we cannot exclude the possibility that the *C. gobio* sample itself was the reason for the false-negative detection, as it might not have contained *C. gobio* DNA in sufficient concentration or due to sampling or laboratory errors, such as specimen misidentification, a swab inaccurately taken or DNA degradation.

The striped ruffe (*Gymnocephalus schraetser*) only has 13 reference sequences available in the Midori2 database, none of which is a 12S sequence. Consequently, the lack of reference for the 12S marker prevents a species level assignment for the tele02, MiFish-U, and 12SV5 primer pairs. However, all 12S primer pairs included OTUs assigned to *Gymnocephalus* that were trimmed to genus level due to low reference similarity threshold (< 97%). While no primer pair was able to detect *G. schraetser*, the SeaDNA-mid COI primer contained one ambiguous OTU assigned to *G. schraetser/cernua*. Thus, it remains unclear if the stripped ruffe can be distinguished from *G. cernua*, using eDNA primer pairs.

Furthermore, various species are known to be indistinguishable with the short target fragment lengths used for eDNA metabarcoding. Particularly the two common lamprey species *Lampetra fluviatilis* and *L. planeri* could not be distinguished with any of the used primer pairs. The species status of these two 'sister species' has puzzled scientists for decades and while a genome-wide divergence can be observed (Mateus et al. 2013), they are known to share mitochondrial haplotypes (Espanhol et al. 2007). Considering that most eDNA primer pairs target short mitochondrial fragments of approximately 180 bp, a distinction of these species with eDNA metabarcoding will most likely not be possible in the foreseeable future.

The zingel (*Zingel zingel*) was not detected by any primer pair despite the availability of COI and sRNA reference sequences in the Midori2 database. The closely related Danube zingel (*Zingel streber*) has various COI and 16S reference sequence available and was detected by the SeaDNA-mid and LH16S primer pair. Thus, the most likely explanation for the absence of *Zingel zingel* is errors in sampling or laboratory handling that led to the sample failure.

Ambiguous detections

In several instances, the distinction between true-positive, false-positive, and false-negative detections were very narrow. For several species, we observed misidentification with closely related species, which resulted in false-positive and false-negative assignments in single cases. For example, a species that was not detected by any primer pair is *Rutilus pigus*, the Danube roach. This species is closely related to the cactus roach (*R. virgo*) which was once considered a subspecies (*Rutilus pigus* subsp. *virgo* (Heckel, 1852)) and occurs in the same habitats. However, since molecular data showed that *R. pigus* and *R. virgo* are separate species (Pourshabanan et al. 2022), either the reference taxonomy is incorrect, which can occur in a non-curated database such as Genbank, or the specimen that was sampled for the mock community was actually *R. virgo*. For both species COI reference sequences are available in the Midori2 database, however, no 12S or 16S reference sequences are present in v249. However, in Midori2 v255 nine 12S reference of which respectively at least four are matching with the 12SV5, MiFish-U, and tele02 primer pair. In our dataset, the tele02

(1 OTU, 96.5%) and MiFish-U (1 OTU, 96.0%) both detected OTUs assigned to the genus *Rutilus*, besides *Rutilus rutilus* (which was present in the mock community), rendering these false-negative assignments as result of missing 12S reference sequences. These cases of false-negative or ambiguous assignment can easily be fixed by closing gaps in the reference database. Furthermore, the false-positive *Rutilus virgo* assignment by the SeaDNA primer pair was most likely not a false-positive detection due to primer bias or lack of reference sequences but rather a lack of species name harmonisation or misidentification.

For the European mudminnow (*Umbra krameri*), only four reference sequences (for 12S, COI, 16S) are available in the Midori2 v249 database and it was not detected by any primer pair in our study. According to the Midori2 database assessment the SeaDNA-mid, LH16S, and 12SV5 primer pairs could have detected *U. krameri*, as suitable reference sequence are available. Here, the SeaDNA-mid and 12SV5 primer pairs false-positively detected the closely related species *Umbra pygmaea* and the teleo2, MiFish-U, and LH16S detected *Umbra limi/pygmaea*. Both *U. limi* (Central mudminnow) and *U. pygmaea* (Eastern mudminnow) are native to North America, and particularly the latter has been introduced to Western and Central Europe. One explanation for the incorrect assignments could be a misidentification of the specimen from which the mucus sample was taken. If so, the specimen identified as European mudminnow was truly an invasive Eastern mudminnow. This case should be further investigated since the European mudminnow is listed as 'vulnerable' (IUCN Red List of Threatened Species in 2010) and should ideally be distinguishable from the invasive Eastern mudminnow with eDNA metabarcoding.

Furthermore, we observed several cases of "difficult" taxonomic assignments. Here, particularly OTUs assigned to the genera *Hucho*, *Sander* and *Leuciscus* caused ambiguities. The Danube salmon (*Hucho hucho*) was initially only detected by the SeaDNA-mid and LH16S primer pairs. The three 12S primer pairs faced ambiguities caused by hits to the Sichuan taimen (*Hucho bleekeri*) and the Siberian taimen (*Hucho taimen*), which all share identical 12S sequences. However, since the Danube salmon is the only present species of the genus *Hucho* in Central Europe, *H. bleekeri* and *H. taimen* were ruled out for the tele02, MiFish-U and 12SV5 primer pairs. Similarly, the pikeperch (*Sander lucioperca*) is geographically clearly separated from the sauger (*S. canadensis*), but the two species are not genetically distinguishable with the investigated markers, leading to flag 2 ambiguities ("Two species, one genus"). In this case, however, based on the current distribution ranges, one can account for this ambiguity, similarly to the Danube salmon. Nevertheless, if one of the *Hucho* or *Sander* species were to be introduced to Central Europe, not all primer pairs could distinguish the native species, which could be of concern for invasive species monitoring. The common dace (*Leuciscus leuciscus*) and ide (*L. idus*), however, are highly prone to causing flag 1 ambiguities. This can be caused by several reasons: for instance, species of the family Leuciscidae are known to commonly hybridize, such as the bleak (*Alburnus alburnus*) and chub (*Leuciscus cephalus*) (Wheeler 1978) or chub and roach (*Rutilus rutilus*) (Wheeler and Easton 1978). This can lead to mitochondrial introgression, causing reference sequences of different species to be identical. Another reason is the wide distribution of common dace across Europe and its habitus typical for the family Leuciscidae. This can result in false species identification that is propagated to incorrect database entries, which ultimately can lead to ambiguous assignments. Here, a sophisticated curation of the Midori2 database, or the usage of a custom reference database,

including reference sequences from a known source, might help to reliably distinguish *L. leuciscus* and *L. idus*. Another reason for false-negative assignments may occur in the automated taxonomic assignment of OTUs due to unclear species status or the use of synonyms. For example, we were aware from previous eDNA metabarcoding datasets that *Rhodeus amarus* and *R. sericeus* are used synonymously and we corrected our dataset for this issue (*Rhodeus amarus/sericeus*).

While in this study we used the Midori2 database, which is a curated version of the Genbank database, another widely used reference library for mitochondrial sequences is the MitoFish database (Sato et al. 2018). While reference sequences for most fish are available in the MitoFish database, some species cannot be assigned due to the absence of e.g., whole genome sequences (e.g., *Romanogobio albipinnatus*). Additionally, the comparably lower overall number of reference sequences might be of concern in light of intraspecific variation and could lead to false-negative assignments.

False-positive assignments

The detection of false-positives is of particular concern since it drastically reduces the robustness of taxa lists. Particularly the less specific vertebrate primer pairs were prone to produce comparably high numbers of false-positive assignments. Here, 12SV5 and LH16S were the only datasets that included marine fish taxa, which were not present in the mock community of Central European freshwater fish. Since no marine samples have been processed in this laboratory, cross-contaminations can be ruled out. The most likely explanation for these false-positive assignments is the placement of target fragments in conserved regions to amplify a broader taxonomic range (e.g., vertebrates). However, this will ultimately decrease the taxonomic resolution for specific taxa within that group (e.g., fish species). For the here investigated primer pairs most likely the short fragment length (12SV5 primer pair; 106 bp) or the fragment location for the LH16S primer pair the number of substitutes is too low for reliable fish identification.

Furthermore, incorrect assignments of closely related species were observed for the less specific vertebrate primer pairs 12SV5 and LH16S. These included the Asian *Chondrostoma prespense* instead of *C. nasus*, the North American *Thymallus arcticus* instead of *T. thymallus*, or *Pungitius platygaster* instead of *P. pungitius*. Again, the conserved regions amplified by the 12SV5 and LH16S primer pairs could have led to these false-positive assignments. Particularly phylogenetically 'young' species that have not been separated long and e.g., share mitochondrial haplotypes (Espanhol et al. 2007) or closely related species that exhibit hybridisation and introgression (Hata et al. 2019; De Santis et al. 2021) are potentially not distinguishable with short and conserved target fragments.

However, also the tele02, MiFish-U and SeaDNA-mid primer pairs showed false-positive assignments. Even though the asp (*Leuciscus aspius*) was not included in the mock community, it was detected by all three primer pairs. Since it was consistently detected by the tele02 (2 OTUs, 98% similarity to reference sequence, 8578 reads, 10/10 samples), MiFish-U (2 OTUs, 98%, 7246 reads, 10/10 samples), and the SeaDNA primer pair (1 OTU, 100%, 156 reads, 9/10 samples), the most likely explanation for the detection of *L. aspius* is a misidentification during sampling (e.g., another closely related cyprinid species).

Another explanation is that the DNA of one species can be found in the mucus of another species' mucus, which could potentially also contain eDNA traces from other fish that were present during sampling. Another case of false-positive detection is the Japanese huchen (*Parahucho perryi*), which was detected in low read abundances by the tele02 primer pair (1 OTU, 98%, 114 reads, 9/10 samples). The Japanese huchen is not recorded from Central Europe but is related to both the huchen (*Hucho hucho*) and brown trout (*Salmo trutta*), which were both present in the mock community. The most likely explanation is that this false-positive assignment originates from huchen or brown trout DNA that is amplified by the tele02 primer pair followed by misassignment. The low read abundance observed in this dataset and its occurrence in combination with the brown trout in other eDNA metabarcoding datasets using the tele02 primer pair (Macher et al. unpublished data) hints towards a systematically false-positive detection of the Japanese huchen in the presence of the brown trout. A similar case is the detection of the Asian sharp-snouted lenok (*Brachymystax lenok*) with the MiFish-U primer pair, which is a salmonoid species related to trouts.

While most ambiguous taxonomic assignments and false-positive detections can be easily corrected using further information (e.g., species distribution), primer pairs that are not prone to false-positive assignments, such as the tele02, MiFish-U and the SeaDNA-mid primer pairs, are to be preferred over the less specific 12SV5 and LH16S primer pairs when investigating fish communities based on eDNA metabarcoding.

Conclusion

In conclusion, our study highlights how the choice of primer has a major effect on the outcome of eDNA metabarcoding analysis. Among the investigated primer pairs, the tele02 primer pair was the best choice for eDNA metabarcoding of Central European freshwater fish, showing the highest detection ability and good reproducibility with the fewest false-positive and false-negative detections. We also observed that gaps in reference libraries can still lead to false-negative detections and thus should be addressed. Through careful selection of the primer pair, laboratory protocol, and bioinformatic pipeline, eDNA metabarcoding is becoming an increasingly reliable tool for fish monitoring.

Disclaimer

The collection of mucus samples is not categorized as an animal experiment and did not require further authorisation. All sampling events were coordinated with local authorities. Fish specimens were solely caught during sampling events for monitoring campaigns and were handled by experts.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

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Author contributions

Till-Hendrik Macher: Conceptualization, Methodology, Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing; Robin Schütz: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing; Atakan Yildiz: Methodology, Writing – review & editing; Arne J. Beermann: Conceptualization, Validation, Supervision, Writing – review & editing; Florian Leese: Conceptualization, Resources, Supervision, Project administration, Funding acquisition, Writing – review & editing.

Author ORCIDs

Till-Hendrik Macher https://orcid.org/0000-0001-6164-9557
Robin Schütz https://orcid.org/0000-0002-4349-6697
Atakan Yildiz https://orcid.org/0009-0007-0333-9877
Arne J. Beermann https://orcid.org/0000-0003-0403-0322
Florian Leese https://orcid.org/0000-0002-5465-913X

Data availability

The raw data were deposited at the European Nucleotide Archive (https://www.ebi.ac.uk/ena/browser/home) under the accession number PRJEB60937.

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Supplementary material 1

Pairwise comparison of the log-transformed reads of the non-normalized mock community (MC1) compared to the DNA concentration (ng/ul) of each species

Authors: Till-Hendrik Macher, Robin Schütz, Atakan Yildiz, Arne J. Beermann, Florian Leese

Data type: png

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Link: https://doi.org/10.3897/mbmg.7.103856.suppl1

Supplementary material 2

Pairwise comparison of the log-transformed reads of the non-normalized mock community (MC1) compared to log-transformed reads of the normalized mock community (MC2) of each species

Authors: Till-Hendrik Macher, Robin Schütz, Atakan Yildiz, Arne J. Beermann, Florian Leese

Data type: png

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Link: https://doi.org/10.3897/mbmg.7.103856.suppl2

Supplementary material 3

Sampled specimens and their respective species assignment collected for the fish mock community, extraction date, collection site, and concentration after DNA extraction

Authors: Till-Hendrik Macher, Robin Schütz, Atakan Yildiz, Arne J. Beermann, Florian Leese

Data type: xlsx

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Link: https://doi.org/10.3897/mbmg.7.103856.suppl3

Supplementary material 4

List of all species reported from Germany, their occurrence status, and their presence in the mock community (data from fishbase.org)

Authors: Till-Hendrik Macher, Robin Schütz, Atakan Yildiz, Arne J. Beermann, Florian Leese

Data type: xlsx

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Link: https://doi.org/10.3897/mbmg.7.103856.suppl4

Supplementary material 5

List of all ambiguous assignments

Authors: Till-Hendrik Macher, Robin Schütz, Atakan Yildiz, Arne J. Beermann, Florian Leese

Data type: xlsx

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Link: https://doi.org/10.3897/mbmg.7.103856.suppl5

Supplementary material 6

List of over splitting rates per primer pair for each detected species

Authors: Till-Hendrik Macher, Robin Schütz, Atakan Yildiz, Arne J. Beermann, Florian Leese

Data type: xlsx

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Link: https://doi.org/10.3897/mbmg.7.103856.suppl6

Supplementary material 7

Protocol for the adapted NucleoMag Tissue Kit

Authors: Till-Hendrik Macher, Robin Schütz, Atakan Yildiz, Arne J. Beermann, Florian Leese

Data type: docx

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Link: https://doi.org/10.3897/mbmg.7.103856.suppl7

Supplementary material 8

Unmodified TaXon tables of each primer pair

Authors: Till-Hendrik Macher, Robin Schütz, Atakan Yildiz, Arne J. Beermann, Florian Leese

Data type: zip

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Link: https://doi.org/10.3897/mbmg.7.103856.suppl8

Supplementary material 9

Processed TaXon tables of each primer pair (subtracted negative controls and filtered for fish and lamprey taxa OTUs)

Authors: Till-Hendrik Macher, Robin Schütz, Atakan Yildiz, Arne J. Beermann, Florian Leese

Data type: zip

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Link: https://doi.org/10.3897/mbmg.7.103856.suppl9

Supplementary material 10

Processed and manually curated TaXon tables of each primer pair

Authors: Till-Hendrik Macher, Robin Schütz, Atakan Yildiz, Arne J. Beermann, Florian Leese

Data type: zip

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Supplementary material 11

Python scripts used in this study

Authors: Till-Hendrik Macher, Robin Schütz, Atakan Yildiz, Arne J. Beermann, Florian Leese

Data type: py

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